THE JOURNAL OF IMMUNOLOGY

Lymph Node Hypertrophy following *Leishmania major* Infection Is Dependent on TLR9

Lucas P. Carvalho, Patricia M. Petritus, Alyssa L. Trochtenberg, Colby Zaph, David A. Hill, David Artis and Phillip Scott

J Immunol 2012; 188:1394-1401; Prepublished online 28 December 2011;
doi: 10.4049/jimmunol.1101018
http://www.jimmunol.org/content/188/3/1394

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Lymph Node Hypertrophy following *Leishmania major* Infection Is Dependent on TLR9

Lucas P. Carvalho,*,†,[1] Patricia M. Petritus,*,†,[1] Alyssa L. Trochtenberg,* Colby Zaph,*,[2] David A. Hill,*,† David Artis,*,† and Phillip Scott*

Control of the protozoan parasite *Leishmania major* is dependent on establishing a robust T cell response. An early event in the development of an effective T cell response is the expansion (or hypertrophy) of the lymph node draining the site of infection, although the mechanisms involved in this response are not completely understood. In this study, we show that lymph node hypertrophy following *L. major* infection in mice is associated with increased recruitment of lymphocytes to the lymph node from the blood, and that CD62L-deficient mice, which are unable to recruit cells to the lymph node, develop a chronic infection with *L. major*. Injection of *L. major*-activated dendritic cells promoted lymph node hypertrophy, and this correlated with an increase in the expression of CCR7 on dendritic cells, although the upregulation of CCR7 occurred on the bystander (uninfected) dendritic cells rather than those containing parasites. We found that increased CCR7 expression was TLR9-dependent, that TLR9$^{-/-}$ dendritic cells migrated less efficiently to the draining lymph node, and that TLR9$^{-/-}$ mice exhibited a deficit in lymph node expansion following *L. major* infection, as well as increased susceptibility. Taken together, to our knowledge, these results are the first to demonstrate that activation of dendritic cells via TLR9 is essential for the induction of lymph node hypertrophy in leishmaniasis. ***The Journal of Immunology*****, 2012, 188: 1394–1401.**

Leishmania major is a protozoan parasite that is controlled by CD4$^+$ Th1 and CD8 T cells (1). The magnitude of this immune response is regulated by several factors, including the parasite load, the induction of regulatory mechanisms, and the frequency of Ag-reactive cells. Because the frequency of a naive T cell with any particular specificity is low, the immune system depends upon the ability of naive T cells to recirculate throughout secondary lymphoid organs, thus enhancing the potential for an appropriate T cell to come in contact with its cognate Ag. Following infection, this pathway is enhanced by the rapid expansion (or hypertrophy) of the lymph nodes (LNs) draining the site of infection (2–6). Not only does this response promote increased entry of naive T cells, but also allows entry of effector T cells not normally able to enter LNs (7). The generation of a robust protective immune response to *L. major* is associated with substantial hypertrophy of the draining LN (dLN), although the factors that initiate LN expansion in leishmaniasis are not defined (8).

The importance of this response in leishmaniasis is suggested by several observations. First, mice that have been engineered to express high levels of CCL21, which leads to CCR7 downregulation and therefore inhibits T cell entry into the LNs, develop a chronic disease following infection with *L. major* (9). Studies in mice lacking LNs highlight the importance of LNs in resistance, as these mice developed an increased susceptibility to *L. major* (10). We recently correlated minimal LN expansion with the susceptibility and limited immune responses seen in mice infected with *Leishmania mexicana*, further suggesting the importance of this physiologic response in establishing effective immunity (8). Finally, in addition to being protective, an exaggerated expansion of the LN can be associated with disease. For example, substantial lymphadenopathy often occurs in patients infected with *Leishmania braziliensis* parasites, and although these individuals eliminate most of their parasites, they have a tendency to develop severe inflammatory lesions, sometimes with chronic metastatic mucosal disease (11, 12). These observations raise the question of how LN expansion is initiated and regulated following *Leishmania* infection.

Recent studies have begun to elucidate the mechanisms responsible for LN expansion following administration of adjuvants (2–5). Dendritic cells (DCs) play a critical role in initiating the response, as injection of activated DCs into the skin induces hypertrophy in the dLN and depleting DCs abrogates adjuvant-induced LN expansion (2, 4). Once DCs initiate this response it leads to enhanced recruitment of cells from the blood to the dLN and it increases cellularity. Enhanced recruitment is likely mediated by increased extravasation of cells from the blood into the LN through high endothelial venules expressing higher levels of chemokines capable of binding CCR7 on T cells, such as CCL21. In the next several days vascular endothelial growth factor-dependent endothelial cell proliferation results in increased vascularity, specifically increasing the number of high endothelial venules through which cells gain entry to LNs. Concomitantly, there is a DC- and vascular endothelial growth factor-dependent increase in the lymphatic vessels within the LN (lymphangiogenesis) and in the tissues draining the site of inflammation (13, 14), which further increases entry of cells (such as DCs) from the tissues into the LN. Less is known about the resolution of LN

*Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104; and †Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

[1]L.P.C. and P.M.P. contributed equally to this work.

[2]Current address: Biomedical Research Centre and Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada.

Received for publication April 8, 2011. Accepted for publication November 23, 2011.

This work was supported by National Institutes of Health Grant AI076257 (to P.S.) and Training Grant AI055400-06 (to P.M.P.).

Address correspondence and reprint requests to Dr. Phillip Scott, Department of Pathobiology, School of Veterinary Medicine, Room 310 Hill Pavilion, 380 South University Avenue, Philadelphia, PA 19104-4539. E-mail address: pscott@vet.upenn.edu

Abbreviations used in this article: DC, dendritic cell; dLN, draining lymph node; LN, lymph node; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101018

hypertrophy, although recently DCs have also been shown to play a role in stabilizing the vasculature following expansion (4).

In this study, we address whether the LN hypertrophy we see following *L. major* infection is associated with increased cell recruitment, and what innate immune responses are involved in the response. We found that LN hypertrophy following *L. major* infection is associated with a substantial increase in cell recruitment throughout the first several weeks of infection, that *L. major*-activated DCs induce this response, and that TLR9 is required for the rapid *L. major*-induced LN hypertrophy seen postinfection.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from the National Cancer Institute (Fredricksburg, MD). TLR9-deficient mice were obtained from Dr. Larry Turka (University of Pennsylvania). Female B6;129S2-*Sell*^{tm1Hyn}/J (CD62L-deficient mice) were obtained from The Jackson Laboratory. Female B6129 mice obtained from The Jackson Laboratory were used as a wild-type (WT) control for the experiments with the CD62L-deficient mice. Germ-free mice were maintained in a colony at the University of Pennsylvania. Animals were housed in a specific pathogen-free environment and tested negative for pathogens in routine screening. All experiments were conducted following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Parasites and infections

L. major V1 parasites (MHOM/IL/80/Friedlin) or *L. major* V1 parasites expressing red fluorescence protein (DsRed *L. major*) (15) were grown until stationary phase in Grace's insect culture medium (Life Technologies, Gaithersburg, MD) supplemented with 20% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and metacyclic promastigotes isolated by density gradients (16). For infection of mice, 1–2 $\times$ 10^6 metacyclic parasites were injected into the footpad or ear, and the course of infection was monitored by measuring lesion size. Parasites were quantitated by limiting dilution.

Bone marrow-derived DCs

Bone marrow-derived DCs were cultured as previously described (17). Briefly, bone marrow DC precursors were differentiated for 8–10 d in the presence of 20 ng/ml GM-CSF in RPMI 1640 containing 10% FBS, 100 U/ml penicillin/streptomycin, 0.05 mM 2-ME, and 2 mM L-glutamine. On days 8–10 of culture DCs were harvested and infected with promastigotes at a 10:1 or 5:1 ratio. After 3 h cells were washed twice for elimination of extracellular parasites and DCs were incubated for 18 h at 37˚C. After an incubation period of 18 h at 37˚C, DCs were harvested and stained for flow cytometry as described below.

CCL21 ELISA

dLNs were harvested on days 3, 7, and 14 following injection of C57BL/6 mice with *L. major* or injection of PBS. The LNs were disrupted using a tissue lyser with 5-mm beads in PBS and protease inhibitor mixture (Sigma-Aldrich). The presence of CCL21 was assessed by ELISA (R&D Systems).

Cytokine mRNA responses

Total RNA was extracted from homogenized dLN tissue with RNeasy columns, following the manufacturer's instructions (Qiagen). The cDNA was generated using SuperScript II reagents (Invitrogen Life Technologies). Real-time PCR for IFN-γ and IL-4 were performed using SYBR Green primers (Qiagen). β-actin was used as a control.

Flow cytometry

For flow cytometry, DCs were harvested, stained with fluorochrome-conjugated Abs for surface markers (CD11c, CD11b, MHC class II, CCR7, CD45.1, and CD45.2; e-Bioscience), and fixed by using 2% formaldehyde. Samples were acquired on a FACSCanto flow cytometer (BD Pharmingen), and analysis was performed using FlowJo software (Tree Star). The cells were gated based on the live cell gate and then further gated on the CD11b$^+$CD11c$^+$ population.

Lymphocyte homing into dLNs

To assess cell recruitment into LNs draining the site of infection, naive mice were sacrificed and a single-cell suspension of spleen cells was prepared and labeled with CFSE. Splenocytes (5×10^6) were injected i.v. into mice that had previously been infected with *L. major* for various periods of time. After 18 h the mice were sacrificed, the LNs draining the site of infection, as well as the contralateral LNs (nondraining), were harvested, and the number of cells was quantitated. The presence of the donor cells was assessed in the dLNs by analyzing the CFSE-positive cells. For these experiments the CFSE label was used only to identify the transferred cells.

Statistics

Statistical analysis was performed using a two-tailed Student *t* test. Differences were considered significant at $p < 0.05$.

Results

LN hypertrophy following L. major *infection involves increased recruitment of cells to the dLN*

Within a few days of infection with *L. major* there is a substantial increase in the cellularity of the LN draining the site of infection, although a visible lesion is not evident for several weeks (Fig. 1*A*). The dLN continues to increase in cellularity throughout the course of infection, reaching 30- to 40-fold normal size (Fig. 1*B*). Although proliferation of lymphocytes in the dLNs likely contributes to the increase in LN cellularity, we were interested to know whether increased recruitment of cells from the blood into the LNs was also occurring. Increased recruitment of T cells would promote a better immune response by increasing the chances that a specific T cell would come into contact with DCs presenting leishmanial Ags. T cells are able to migrate into LNs owing to the expression of CD62L and the chemokine receptor CCR7. CCR7 binds to CCL21 or CCL19, both of which are expressed on the endothelium. Consistent with the role of CCL21, we found that associated with *L. major*-induced LN hypertrophy was an increase in CCL21 protein levels (Fig. 1*C*), which would promote increased entry of cells into the dLNs.

To directly assess cell migration into LNs, we adoptively transferred CFSE-labeled lymphocytes into mice that had been previously infected with *L. major* at various times. After 18 h we sacrificed the recipients, harvested the dLN and the contralateral (nondraining) LN, and quantified the recruited donor cells by flow cytometry (Fig. 1*D*). Within the first 24 h of infection, there was a significant increase in the number of donor cells recruited to the dLN as compared with the non-dLN (Fig. 1*E*). To determine whether the increased recruitment was transient, we examined several time points following infection and found that increased cell recruitment was evident throughout the first 4 wk infection (Fig. 1*F*). Although there was a change in the ratio of T and B cells in the dLN over time, presumably due to B cell proliferation, there was no preferential recruitment of T or B cells (data not shown). Taken together, these data indicate that *L. major* induces a rapid increase in dLN cellularity that is in part mediated by a sustained increase in the recruitment of lymphocytes to the dLN.

Previous studies suggested that the absence of LNs leads to increased susceptibility to *L. major* (9, 10). We infected CD62L-deficient mice on a C57BL/6 background to confirm these observations. CD62L is expressed on the surface of cells and is required for the efficient entry of lymphocytes into the LN via the high endothelial venules (18). Transient blockade of CD62L inhibits the development of Th2 responses in *L. major*-infected BALB/c mice, rendering them more resistant (19). In contrast, when LN hypertrophy was completely blocked by infection of CD62L knockout mice, we found that CD62L-deficient mice exhibited a relatively normal course of infection for the first few weeks of infection, but these mice were unable to resolve their infections (Fig. 2*A*). They maintained chronic lesions for >20 wk postinfection. Whereas parasites appeared to be cleared in the

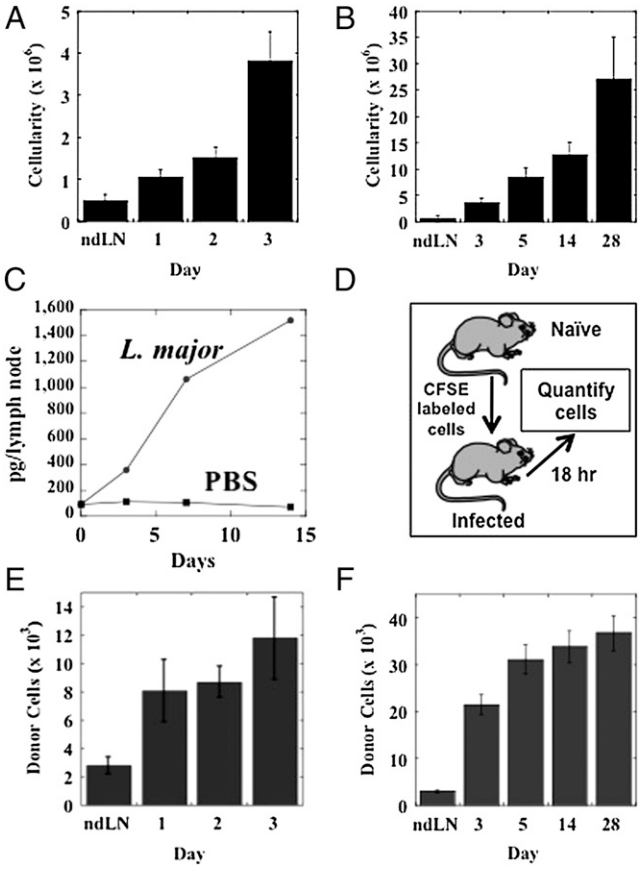

FIGURE 1. *L. major* induces increased recruitment of cells to the dLN. C57BL/6 mice were infected with *L. major* and the cellularity of the dLN was assessed from day 1 to 3 (*A*) or from day 3 to 28 (*B*). These responses are compared with the cellularity of nondraining LNs. LNs were harvested from *L. major* or PBS-injected mice at days 3, 7, and 14. The cells were dissociated, and CCL21 levels were assessed by ELISA (*C*). Splenocytes from naive C57BL/6 mice were CFSE labeled and injected i.v. in mice that had previously been infected with *L. major*, and after 18 h the number of CFSE-labeled cells was quantitated by flow cytometry in the LN draining the site of infection (*D*). Quantitation of donor cells was done during the course of *L. major* infection from day 1 to 3 (*E*) or from day 3 to 28 (*F*). The number of cells migrating into the nondraining LNs did not change significantly during the course of the experiment, and the average is shown. The data shown are representative of two or more experiments. ndLN, nondraining LNs.

lesions of the control mice, the CD62L-deficient mice maintained $\sim 10^3$ parasites in their lesions (data not shown). As expected, the dLNs were correspondingly smaller in the CD62L-deficient mice both at 5 and 20 wk following infection (Fig. 2*B*).

L. major-*activated DCs induce LN hypertrophy*

Activation of DCs in the skin induces hypertrophy in the dLN. Thus, when LPS-matured DCs are injected into the skin they induce an increase in the cellularity of the dLN (2, 4). Therefore, we asked whether DCs exposed to *L. major* would also induce LN hypertrophy when injected into the skin. Bone marrow-derived DCs were infected in vitro with *L. major* metacyclic promastigotes, leading to ~30–35% of the DCs containing *L. major* parasites. We injected the DCs exposed to the parasites into the skin, and 3 d later we assessed the cellularity of the dLN. We found a significant increase in the cellularity of those dLNs from mice injected with cultures of DCs that had been exposed to *L. major* (Fig. 3). These results indicate that *L. major* stimulates a population of DCs that acquire the capacity to induce LN hypertrophy.

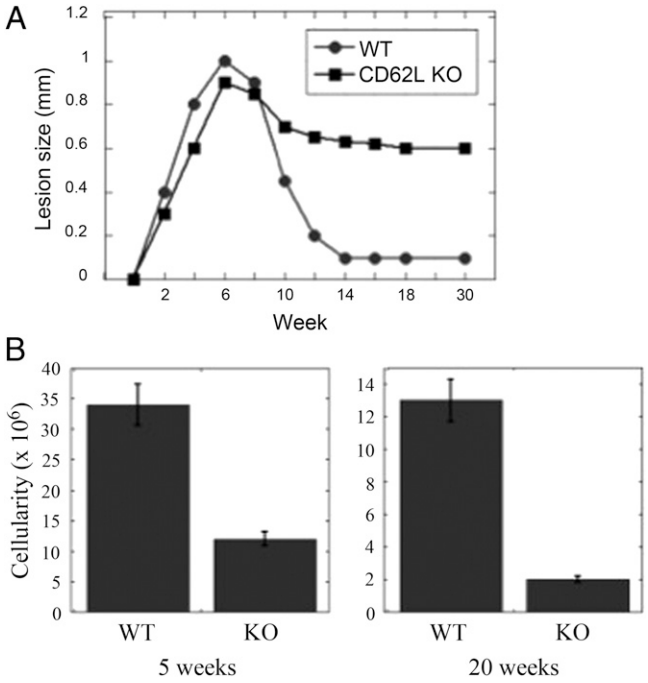

FIGURE 2. *L. major* infections in CD62L-deficient mice fail to resolve their lesions. *A*, B6129 (WT) or CD62L$^{-/-}$ mice were infected with *L. major* and the course of infection was monitored. The results expressed are the mean (±SEM) lesion size of five mice per group. *B*, The cellularity of the dLN was assessed at weeks 5 and 20. The results expressed are the mean (±SEM) number of cells in the dLN of three mice per group. The data shown are representative of two experiments.

L. major *induces CCR7 expression in DCs in a TLR9-dependent manner*

A key factor in the development of LN hypertrophy is the ability of DCs to become activated and to migrate from the tissues to the dLN, a process that is dependent on the expression of the chemokine receptor CCR7 (2, 20). Once in the dLN, DCs promote LN expansion by directly or indirectly promoting increased blood flow to the LN. Therefore, we hypothesized that following *L. major* infection DCs become activated and are licensed to promote LN hypertrophy. To test this hypothesis we examined expression of MHC class II and CCR7 on infected DCs. We found substantial upregulation of CCR7 following *L. major* infection (Fig. 4).

Although we observed an increase in MHC class II and CCR7 in cultures of DCs infected with *L. major*, we previously reported that *Leishmania*-induced DC activation occurs primarily in the bystander cells within the culture, that is, the cells that are not

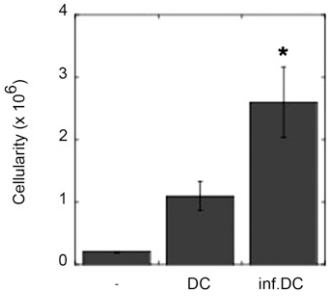

FIGURE 3. DCs activated by *L. major* induce LN hypertrophy. Bone marrow-derived DCs were infected with *L. major*, and 5×10^5 DCs or uninfected DCs were injected into the footpad of C57BL/6 mice, and the dLNs were harvested 3 d later. The results are the mean ± SEM of three mice per group and are representative of two experiments. *$p < 0.5$.

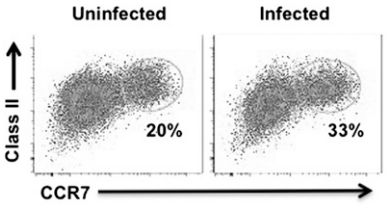

FIGURE 4. *L. major* infection induces increased CCR7 expression on DCs. Bone marrow-derived DCs were infected at a 10:1 ratio with *L. major* for 2 h and then cultured overnight. Cells were stained with fluorescent Abs and results were assessed by flow cytometry. These results are representative of three experiments.

infected (21). To determine whether this was the case for CCR7 expression, we infected DCs with DsRed *L. major*, which allowed us to distinguish the infected and the uninfected cells within the same culture. Consistent with our previous observations, we found that upregulation of CCR7 occurred in the bystander DCs, but not in the infected DCs (Fig. 5*A*, 5*B*).

Previous studies found that *Leishmania* parasites activate DCs via TLR9 (22–24). We confirmed this by stimulating DCs with *Leishmania* DNA and assessing the production of cytokines. *Leishmania* DNA stimulated the production of IL-12 and TNF-α in WT DCs, but not in TLR9$^{-/-}$ DCs (data not shown). Therefore, because DC activation is required for LN hypertrophy, we hypothesized that TLR9 may similarly be critical, and investigated whether the *L. major*-induced CCR7 upregulation on DCs was TLR9-dependent. DCs from WT or TLR9$^{-/-}$ mice were infected with DsRed parasites, and the expression of CCR7 was assessed at 24 h. As described above, we found that within cultures of WT DCs infected with *L. major*, there was a significant upregulation of CCR7 by the bystander DCs (Fig. 5*A*, 5*B*). In contrast, when we assessed expression of CCR7 by TLR9$^{-/-}$ cells, we found no significant increased expression of CCR7 (Fig. 5*C*, 5*D*). These results are similar to others where TLR9 promoted CCR7 upregulation in DCs (25, 26).

TLR9 is required for LN hypertrophy

The TLR9-dependent upregulation of CCR7 in DCs suggested that LN hypertrophy may be TLR9-dependent as well. To test this hypothesis we compared the LN cellularity in WT and TLR9$^{-/-}$ mice 3 d following *L. major* infection. As shown in Fig. 6*A*, whereas C57BL/6 mice infected with *L. major* exhibited a significant increase in cellularity by day 3, *L. major*-infected TLR9$^{-/-}$ mice exhibited a minimal increase in LN cellularity.

A large and complex microbial community colonizes the skin, respiratory tract, and intestine of mammals, and the influence of these microbes on the host immune system is just beginning to be evaluated (27). Although *L. major* can activate DCs directly, it is possible that commensal-derived signals contribute to DC activation via TLR9. To determine whether commensal-derived signals contribute to dLN hypertrophy, we compared LN hypertrophy induced by *L. major* infection in conventional C57BL/6 or germ-free mice (reared in the absence of live microbial stimuli). We found no significant difference in the cellularity of the dLN in these two groups (Fig. 6*B*). Thus, it appears that commensal-derived signals are not necessary for LN hypertrophy following infection with *L. major*.

Several types of cells express TLR9 and, although we and others have found that *Leishmania* DNA activates DCs (22–24), it was still possible that the in vivo effects we were observing were due to expression of TLR9 on cells other than DCs. To address this issue, we compared the ability of *L. major*-activated WT DCs and TLR9$^{-/-}$ DCs to induce LN hypertrophy. DCs were exposed to *L. major*, and after 24 h the cells were injected into the skin. As seen in Fig. 7*A*, WT DCs promoted an increase in cellularity of the dLN. In contrast, there was no increase evident when DCs from TLR9$^{-/-}$ mice were injected into the skin. The lack of a response by TLR9$^{-/-}$ DCs suggested that *L. major* was unable to appropriately activate TLR9$^{-/-}$ DCs, and thus the DCs would migrate poorly to the dLN. To test this directly, we infected WT and TLR9$^{-/-}$ DCs with *L. major* and inoculated them into the footpad. After 3 d we harvested the dLN and quantitated the donor DCs. As seen in Fig. 7*B*, there were fewer infected TLR9$^{-/-}$ DCs, as compared with infected WT DCs, in the dLN.

Although the results described above indicate that TLR9 expression in DCs is a critical component in the development of LN hypertrophy, it may be that other cells expressing TLR9 contribute to the response. To address this issue, we infected WT DCs and transferred them to either WT or TLR9$^{-/-}$ mice. We found that WT DCs promoted LN hypertrophy in both WT and TLR9$^{-/-}$ mice, indicating that TLR9 expression was only required in the DCs (Fig. 7*C*).

TLR9-deficient mice infected with L. major *exhibit decreased LN hypertrophy for several weeks postinfection*

TLR9-deficient mice exhibit an increased susceptibility to *L. major* infection, and we were interested in determining whether the deficit in LN hypertrophy extended beyond the initial few days of infection (23, 24). We infected C57BL/6 mice or TLR9$^{-/-}$ mice with *L. major* and monitored the course of infection. As reported, TLR9$^{-/-}$ mice developed significantly larger lesions than did control mice, with substantially more parasites when assessed 6

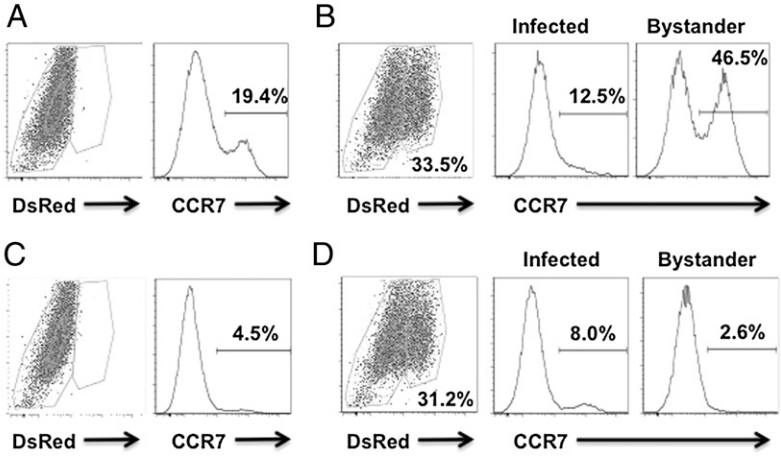

FIGURE 5. *L. major* infection induces increased CCR7 expression on bystander DCs in a TLR9-dependent manner. Bone marrow DC cultures from C57BL/6 (*A*, *B*) or TLR9$^{-/-}$ (*C*, *D*) mice were uninfected (*A*, *C*) or infected (*B*, *D*) at a 10:1 ratio with *L. major* for 2 h and then incubated overnight. Cells were stained with fluorescent Abs and results were assessed by flow cytometry. These results are representative of three experiments.

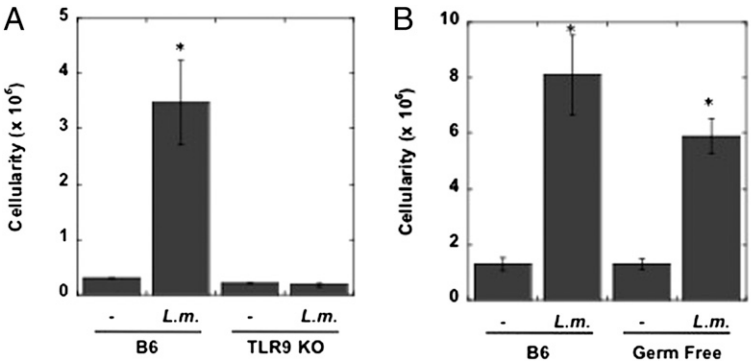

FIGURE 6. TLR9$^{-/-}$ mice exhibit decreased LN hypertrophy following *L. major* infection. C57BL/6 (B6) or TLR9$^{-/-}$ mice were infected with *L. major* or injected with killed *L. major* Ag and the cellularity of the dLN was assessed on day 3 (*A*). Conventional or germ-free C57BL/6 mice were infected with *L. major*, and the cellularity of the dLN was assessed on day 3 (*B*). The results expressed are the mean (±SEM) number of cells in the dLN of three mice per group. The results are representative of two experiments. *$p < 0.5$.

wk following infection (Fig. 8*A*, 8*B*). This increased susceptibility was associated with no significant difference in the IFN-γ mRNA levels between the controls and the TLR9$^{-/-}$ animals (Fig. 8*C*), but there was a significant increase in the levels of IL-4 mRNA in TLR9$^{-/-}$ mice (Fig. 8*D*). To assess whether the absence of TLR9 influenced the ability of *L. major* mice to induce LN hypertrophy over time, we quantitated the cellularity of the dLN during the course of infection. As seen in Fig. 8*E*, the dLNs from TLR9$^{-/-}$ were smaller than those from control mice throughout the first 4 wk of infection. However, by 5 wk, dLN cellularity in WT and TLR9$^{-/-}$ mice was similar and, as previously reported, the TLR9$^{-/-}$ mice were eventually able to resolve their infections (23, 24) (data not shown). Although we do not know what compensates for the absence of TLR9 at these later stages of the infection, we hypothesize that sufficient activated T cells may have slowly accumulated such that through CD40/CD40L interactions DCs become activated. This would be consistent with the findings that CD40 or CD40L is required for resistance to *Leishmania* parasites (28–30).

Discussion

Establishing an immune response is dependent on the ability of a small number of T cells to come in contact with a small number of DCs that are presenting the relevant Ags. Adjuvants increase the chances of a successful interaction between DCs and T cells following immunization, in part by promoting a rapid increase in the cellularity of the dLN. Thus, within the first few days following inoculation of an Ag/adjuvant complex there is an increase in the recruitment of lymphocytes to the LN draining the site of immunization (4, 5, 13). This increased LN cellularity, or LN hypertrophy, requires recognition of pathogen-associated molecules that leads to DC activation. Once activated the DCs migrate to the dLN and orchestrate the response. A similar response is important for establishing immunity to pathogens, and we previously reported that LN hypertrophy occurs rapidly following infection with *L. major* in mice. Indeed, in this study we show that mice failing to exhibit normal LN hypertrophy (due to the absence of CD62L) are unable to resolve their leishmanial lesions. In this study we sought to define the factors involved in *L. major*-induced LN hypertrophy (8). We show that, as in immunization models, early LN hypertrophy is associated with rapid recruitment of cells from the blood to the dLNs following infection, that *L. major* parasites induce upregulation of CCR7 in DCs, and that injection of *L. major*-activated DCs into the skin induces LN hypertrophy. Importantly, we found that TLR9 is critical in mediating these responses. To our knowledge, this is the first study to define the TLR ligand required for *L. major*-induced LN hypertrophy.

The increased recruitment of lymphocytes to the dLN of *L. major*-infected mice was sustained from day 1 through day 28. In contrast, it was recently found that following infection with *Listeria* or lymphocytic choriomeningitis virus mice exhibited a transient decrease in the ability to recruit cells to the LN, in part due to decreased production of CCL21 (31). When we assessed the protein levels of CCL21 in the dLN we saw no evidence of a decrease, and correspondingly, there was no inability to recruit cells to the responding dLNs. Because the deficit in the normal migration of cells in secondary lymphoid organs following *Listeria* or lymphocytic choriomeningitis virus was found to depend on the production of IFN-γ, we hypothesize that *L. major* fails to induce a transient decrease in cell recruitment to the dLN because the parasite induces only a relatively modest IFN-γ response during the first several weeks of *L. major* infection (32).

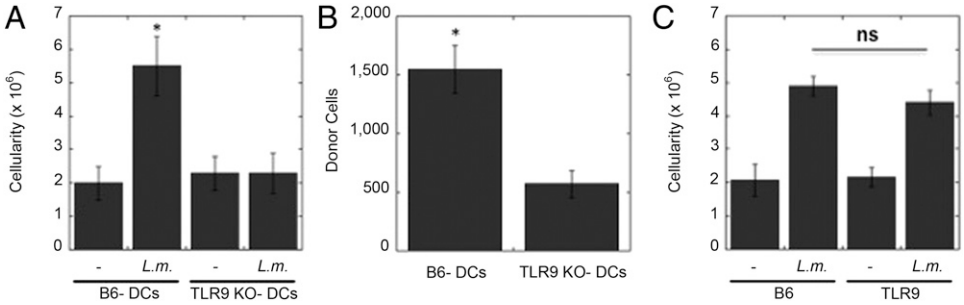

FIGURE 7. TLR9 expression in DCs is required and sufficient to induce LN hypertrophy. TLR9$^{-/-}$ DCs activated with *L. major* failed to induce LN hypertrophy. *L. major*-infected DCs (5×10^5) from C57BL/6 (B6) or TLR9$^{-/-}$ mice, or uninfected DCs, were injected into the footpad of C57BL/6 mice, and the cellularity of the dLN was assessed on day 3 (*A*). *L. major*-infected DCs (1×10^6) from C57BL/6 (B6) or TLR9$^{-/-}$ mice, or uninfected DCs, were injected into the footpad of CD45.1 C57BL/6 mice, and the presence of the donor cells in the dLN was assessed on day 3 (*B*). *L. major*-infected DCs (1×10^6) from C57BL/6 (B6) mice, or uninfected DCs, were injected into the footpad of C57BL/6 mice or TLR9$^{-/-}$ mice, and the cellularity of the dLN was assessed on day 3 (*C*). The results expressed are the mean (±SEM) number of cells in the dLN of three mice per group. The results are representative of two experiments. *$p < 0.5$.

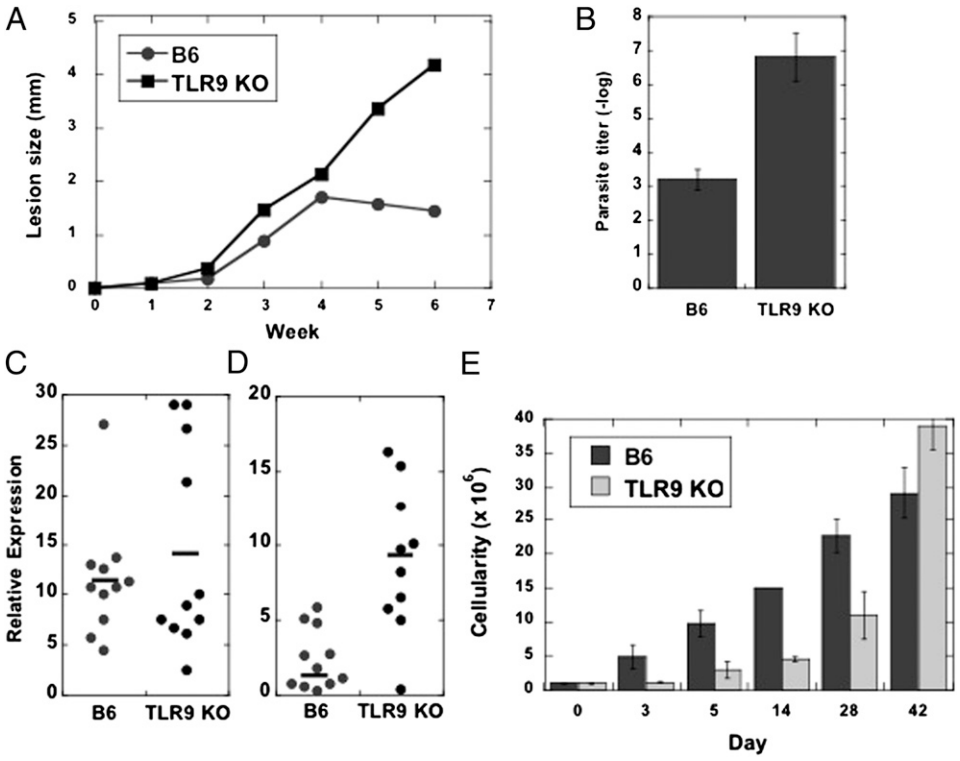

FIGURE 8. TLR9$^{-/-}$ mice are more susceptible to *L. major* and exhibit a deficit in LN hypertrophy for several weeks following infection. *A*, C57BL/6 (B6) or TLR9$^{-/-}$ mice were infected with *L. major* and the course of infection was monitored. The results expressed are the mean (± SEM) lesion size of five mice per group. *B*, At 6 wk the mice were sacrificed and parasites within the lesions were quantitated by limiting dilution. These data are representative of two experiments. *C*, IFN-γ and (*D*) IL-4 mRNA levels were assayed by quantitative RT-PCR on cells from the dLNs of mice sacrificed at 6 wk. Each data point represents one mouse, and the line denotes the average values. IFN-γ levels were not significantly different. IL-4 levels were significantly different ($p < 0.05$). *E*, The cellularity of the dLNs from B6 and TLR9$^{-/-}$ mice were assessed during the course of infection. The data shown are representative of three experiments.

Several studies have established that DCs play a key role in promoting LN hypertrophy following experimental immunization (2–4). Thus, DC activation, and the subsequent CCR7-dependent migration to the dLN of the DCs, is essential to promote LN hypertrophy. Because recent studies show that *Leishmania* parasites, as well as DNA from the parasites, are capable of activating DCs in a TLR9-dependent manner (22–24), we tested whether TLR9 was required for LN hypertrophy. We found that the induction of LN hypertrophy was TLR9-dependent and that the requirement for TLR9 was to promote DC activation, since *L. major* WT but not TLR9$^{-/-}$ DCs induced LN hypertrophy. We also found that the expression of TLR9 on DCs was sufficient to induce LN hypertrophy, as infected WT DCs transferred to TLR9$^{-/-}$ mice led to LN expansion. Finally, we found that commensals associated with the skin were unlikely to contribute to the response since germ-free mice exhibited similar LN hypertrophy following *L. major* infection as conventional mice.

The absence of TLR9 appeared to have an effect on LN hypertrophy for many weeks after the infection, suggesting that the role of TLR9 is not limited to the early interactions between the parasite and the host. Eventually, however, the TLR9$^{-/-}$ mice are able to resolve their lesions, possibly suggesting that other factors compensate as the infection progresses. This might involve other TLRs, since TLR2, 3, 4, and 7 have all been implicated in resistance in various models of leishmaniasis (33–36); however, why these would not compensate for the absence of TLR9 earlier is not clear. An alternative possibility is that as the infection goes on, sufficient activated T cells expressing CD40L accumulate, leading to DC activation via CD40/CD40L interactions. This would be consistent with the requirement for CD40 or CD40L for resolution of *Leishmania* infections in mice (28–30).

CCR7 expression plays an important role in facilitating the migration of cells to the dLN (2, 20, 37). The ability of naive T cells to enter LNs from the blood requires CCR7, and CCR7 on DCs promotes their migration from the tissues to the dLN. Interestingly, however, the requirement for CCR7 differs following infection with different pathogens. *Listeria* infections are still controlled in the absence of CCR7, whereas following *Toxoplasma* infection CCR7-deficient mice die (38). In leishmaniasis, CCR7 appears to be required for the development of an effective immune response. For example, *L. major*-infected CCL21 transgenic mice, which lose CCR7 signaling due to constitutive downregulation, are unable to control the infection (9). Similarly, in visceral leishmaniasis, the absence of the CCR7 ligands CCL19 and CCL21, or the loss of CCR7 on DCs, was correlated with increased pathogenesis, whereas immunotherapy with CCR7-expressing DCs was therapeutic (39, 40). Previous studies have indicated that *L. major* induces CCR7 expression on DCs (41), and our results indicate that the induction of CCR7 by *L. major* is TLR9-dependent, as has been shown in other systems (25, 26). Surprisingly, however, by using DsRed parasites we also found that it was the bystander DCs that upregulated CCR7 expression, rather than the infected DCs. These results are similar to our previous findings showing that bystander DCs, rather than the infected DCs, exhibit increased expression of MHC class II and IL-12, although both the infected and uninfected DCs produced TNF-α (21). Studies are in progress to determine how the bystander DCs become activated, and why infected DCs are unable to upregulate CCR7 expression. However, because many studies have indicated that *Leishmania* can impair signaling pathways in macrophages (42), these results suggest that such impairment is overcome by activating neighboring cells.

Cutaneous leishmaniasis has a wide range of clinical presentations, which depend on the genetics of both the host and the parasite. Patients can develop small lesions that resolve rapidly, chronic single lesions that may take years to heal, and severe metastatic lesions that may result from the lack of a response (diffuse cutaneous leishmaniasis) or an exaggerated immune response (mucosal leishmaniasis). In mice, *L. major* induces a healing lesion in C57BL/6 animals, but a fatal infection in BALB/c mice, whereas *L. mexicana* lesions in C57BL/6 mice fail to resolve (43, 44). We previously found an association between the capacity to induce LN hypertrophy and the chronicity of *L. mexicana* infections in

mice (8). Because *L. mexicana*-infected mice fail to develop a robust Th1 response, we initially reasoned that administration of IL-12 would promote healing, as it does in *L. major* infections. However, administration of IL-12 failed to promote healing, suggesting that the inability of mice to resolve a *L. mexicana* infection was not simply because of the lack of IL-12 (45). This result suggested that in addition to lacking IL-12, there was something else that was defective in *L. mexicana*-infected mice. Because *L. mexicana*-infected mice fail to induce LN hypertrophy, we hypothesize that even when given IL-12, there are too few T cells that are responding to the infection to promote resolution of the lesions. Studies are in progress to determine why *L. mexicana* fails to stimulate LN hypertrophy. However, in preliminary experiments we found no increase in the susceptibility of TLR9$^{-/-}$ mice to *L. mexicana*, suggesting that *L. mexicana* may fail to activate this pathway in vivo, which would account for the inability of *L. mexicana* to promote LN hypertrophy (P. Scott, unpublished observations).

Although failure to develop an optimal immune response can lead to chronic disease, an exaggerated immune response can also lead to severe disease. One example where there is an association between increased LN hypertrophy and the immune response is in patients infected with *L. braziliensis*. Infections caused by *L. braziliensis* are associated with a very strong immune response, involving substantial T cell proliferation and high levels of IFN-γ production (46). Moreover, in a small number of cases the parasites metastasize and the patients develop a severe nonhealing form of the disease known as mucosal leishmaniasis. Interestingly, human *L. braziliensis* infections are often associated with a massive lymphadenopathy, which often is seen prior to the development of the primary lesion (11, 12). Understanding the mechanisms involved in LN hypertrophy is the first step in designing strategies that might lessen the exaggerated and immunopathologic response seen in these patients.

The generation of an optimal immune response depends on ensuring that both the magnitude and type of response are appropriate to the threat. A major focus of research in experimental leishmaniasis has been on ensuring that a Th1-type response is elicited, as Th1 responses are most appropriate to eliminate *L. major* parasites. However, in addition to getting the appropriate response, the magnitude of the response can be just as important in resolving the disease. In human leishmaniasis, chronic disease can result due to either a minimal response that fails to eliminate the parasites or to an exaggerated response where severe inflammation is uncontrolled, such as occurs in mucocutaneous leishmaniasis patients. Because the magnitude of the immune response can be influenced by LN hypertrophy, it is important to better understand how this response is regulated. We think that defining TLR9 as a critical component for LN hypertrophy is a first step in considering how to design approaches focused on modulating the magnitude of the immune response in leishmaniasis.

Acknowledgments

We thank Dr. Larry Turka for the TLR9$^{-/-}$ mice and Dr. Yasmine Belkaid for the DsRed *L. major* parasites. We also acknowledge the technical assistance of Carlos Rodriguez, Sarah Wensley, and Lijun Wen.

Disclosures

The authors have no financial conflicts of interest.

References

1. Scott, P., D. Artis, J. Uzonna, and C. Zaph. 2004. The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development. *Immunol. Rev.* 201: 318–338.
2. Martin-Fontecha, A., S. Sebastiani, U. E. Höpken, M. Uguccioni, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2003. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J. Exp. Med.* 198: 615–621.
3. Angeli, V., and G. J. Randolph. 2006. Inflammation, lymphatic function, and dendritic cell migration. *Lymphat. Res. Biol.* 4: 217–228.
4. Webster, B., E. H. Ekland, L. M. Agle, S. Chyou, R. Ruggieri, and T. T. Lu. 2006. Regulation of lymph node vascular growth by dendritic cells. *J. Exp. Med.* 203: 1903–1913.
5. Soderberg, K. A., G. W. Payne, A. Sato, R. Medzhitov, S. S. Segal, and A. Iwasaki. 2005. Innate control of adaptive immunity via remodeling of lymph node feed arteriole. *Proc. Natl. Acad. Sci. USA* 102: 16315–16320.
6. Ruddle, N. H., and E. M. Akirav. 2009. Secondary lymphoid organs: responding to genetic and environmental cues in ontogeny and the immune response. *J. Immunol.* 183: 2205–2212.
7. Guarda, G., M. Hons, S. F. Soriano, A. Y. Huang, R. Polley, A. Martín-Fontecha, J. V. Stein, R. N. Germain, A. Lanzavecchia, and F. Sallusto. 2007. L-selectin-negative CCR7$^-$ effector and memory CD8$^+$ T cells enter reactive lymph nodes and kill dendritic cells. *Nat. Immunol.* 8: 743–752.
8. Hsu, A. C., and P. Scott. 2007. *Leishmania* mexicana infection induces impaired lymph node expansion and Th1 cell differentiation despite normal T cell proliferation. *J. Immunol.* 179: 8200–8207.
9. Unsoeld, H., K. Mueller, U. Schleicher, C. Bogdan, J. Zwirner, D. Voehringer, and H. Pircher. 2007. Abrogation of CCL21 chemokine function by transgenic over-expression impairs T cell immunity to local infections. *Int. Immunol.* 19: 1281–1289.
10. Ehrchen, J. M., J. Roth, K. Roebrock, G. Varga, W. Domschke, R. Newberry, C. Sorg, C. Müller-Tidow, C. Sunderkötter, T. Kucharzik, and T. W. Spahn. 2008. The absence of cutaneous lymph nodes results in a Th2 response and increased susceptibility to *Leishmania major* infection in mice. *Infect. Immun.* 76: 4241–4250.
11. Barral, A., J. Guerreiro, G. Bomfim, D. Correia, M. Barral-Netto, and E. M. Carvalho. 1995. Lymphadenopathy as the first sign of human cutaneous infection by *Leishmania braziliensis*. *Am. J. Trop. Med. Hyg.* 53: 256–259.
12. Barral, A., M. Barral-Netto, R. Almeida, A. R. de Jesus, G. Grimaldi Júnior, E. M. Netto, I. Santos, O. Bacellar, and E. M. Carvalho. 1992. Lymphadenopathy associated with *Leishmania braziliensis* cutaneous infection. *Am. J. Trop. Med. Hyg.* 47: 587–592.
13. Angeli, V., F. Ginhoux, J. Llodrà, L. Quemeneur, P. S. Frenette, M. Skobe, R. Jessberger, M. Merad, and G. J. Randolph. 2006. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity* 24: 203–215.
14. Liao, S., and N. H. Ruddle. 2006. Synchrony of high endothelial venules and lymphatic vessels revealed by immunization. *J. Immunol.* 177: 3369–3379.
15. Kimblin, N., N. Peters, A. Debrabant, N. Secundino, J. Egen, P. Lawyer, M. P. Fay, S. Kamhawi, and D. Sacks. 2008. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proc. Natl. Acad. Sci. USA* 105: 10125–10130.
16. Späth, G. F., and S. M. Beverley. 2001. A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. *Exp. Parasitol.* 99: 97–103.
17. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
18. Bradley, L. M., S. R. Watson, and S. L. Swain. 1994. Entry of naive CD4 T cells into peripheral lymph nodes requires L-selectin. *J. Exp. Med.* 180: 2401–2406.
19. Laskay, T., I. Wittmann, A. Diefenbach, M. Röllinghoff, and W. Solbach. 1997. Control of *Leishmania major* infection in BALB/c mice by inhibition of early lymphocyte entry into peripheral lymph nodes. *J. Immunol.* 158: 1246–1253.
20. Randolph, G. J. 2001. Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. *Semin. Immunol.* 13: 267–274.
21. Carvalho, L. P., E. J. Pearce, and P. Scott. 2008. Functional dichotomy of dendritic cells following interaction with *Leishmania* braziliensis: infected cells produce high levels of TNF-α, whereas bystander dendritic cells are activated to promote T cell responses. *J. Immunol.* 181: 6473–6480.
22. Schleicher, U., J. Liese, I. Knippertz, C. Kurzmann, A. Hesse, A. Heit, J. A. Fischer, S. Weiss, U. Kalinke, S. Kunz, and C. Bogdan. 2007. NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs. *J. Exp. Med.* 204: 893–906.
23. Liese, J., U. Schleicher, and C. Bogdan. 2007. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur. J. Immunol.* 37: 3424–3434.
24. Abou Fakher, F. H., N. Rachinel, M. Klimczak, J. Louis, and N. Doyen. 2009. TLR9-dependent activation of dendritic cells by DNA from *Leishmania major* favors Th1 cell development and the resolution of lesions. *J. Immunol.* 182: 1386–1396.
25. Asselin-Paturel, C., G. Brizard, K. Chemin, A. Boonstra, A. O'Garra, A. Vicari, and G. Trinchieri. 2005. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *J. Exp. Med.* 201: 1157–1167.
26. Pichyangkul, S., K. Yongvanitchit, U. Kum-arb, H. Hemmi, S. Akira, A. M. Krieg, D. G. Heppner, V. A. Stewart, H. Hasegawa, S. Looareesuwan, et al. 2004. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. *J. Immunol.* 172: 4926–4933.
27. Grice, E. A., H. H. Kong, S. Conlan, C. B. Deming, J. Davis, A. C. Young, G. G. Bouffard, R. W. Blakesley, P. R. Murray, E. D. Green, et al; NISC

Comparative Sequencing Program. 2009. Topographical and temporal diversity of the human skin microbiome. *Science* 324: 1190–1192.
28. Soong, L., J. C. Xu, I. S. Grewal, P. Kima, J. Sun, B. J. Longley, Jr., N. H. Ruddle, D. McMahon-Pratt, and R. A. Flavell. 1996. Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. *Immunity* 4: 263–273.
29. Kamanaka, M., P. Yu, T. Yasui, K. Yoshida, T. Kawabe, T. Horii, T. Kishimoto, and H. Kikutani. 1996. Protective role of CD40 in *Leishmania major* infection at two distinct phases of cell-mediated immunity. *Immunity* 4: 275–281.
30. Campbell, K. A., P. J. Ovendale, M. K. Kennedy, W. C. Fanslow, S. G. Reed, and C. R. Maliszewski. 1996. CD40 ligand is required for protective cell-mediated immunity to *Leishmania major. Immunity* 4: 283–289.
31. Mueller, S. N., K. A. Hosiawa-Meagher, B. T. Konieczny, B. M. Sullivan, M. F. Bachmann, R. M. Locksley, R. Ahmed, and M. Matloubian. 2007. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. *Science* 317: 670–674.
32. Scott, P., A. Eaton, W. C. Gause, X. di Zhou, and B. Hondowicz. 1996. Early IL-4 production does not predict susceptibility to *Leishmania major. Exp. Parasitol.* 84: 178–187.
33. Becker, I., N. Salaiza, M. Aguirre, J. Delgado, N. Carrillo-Carrasco, L. G. Kobeh, A. Ruiz, R. Cervantes, A. P. Torres, N. Cabrera, et al. 2003. *Leishmania* lipophosphoglycan (LPG) activates NK cells through Toll-like receptor-2. *Mol. Biochem. Parasitol.* 130: 65–74.
34. Kropf, P., M. A. Freudenberg, M. Modolell, H. P. Price, S. Herath, S. Antoniazi, C. Galanos, D. F. Smith, and I. Müller. 2004. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major. Infect. Immun.* 72: 1920–1928.
35. Paun, A., R. Bankoti, T. Joshi, P. M. Pitha, and S. Stäger. 2011. Critical role of IRF-5 in the development of T helper 1 responses to *Leishmania donovani* infection. *PLoS Pathog.* 7: e1001246.
36. Flandin, J. F., F. Chano, and A. Descoteaux. 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-γ-primed macrophages. *Eur. J. Immunol.* 36: 411–420.
37. Förster, R., A. C. Davalos-Misslitz, and A. Rot. 2008. CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* 8: 362–371.
38. Noor, S., A. S. Habashy, J. P. Nance, R. T. Clark, K. Nemati, M. J. Carson, and E. H. Wilson. 2010. CCR7-dependent immunity during acute *Toxoplasma gondii* infection. *Infect. Immun.* 78: 2257–2263.
39. Ato, M., A. Maroof, S. Zubairi, H. Nakano, T. Kakiuchi, and P. M. Kaye. 2006. Loss of dendritic cell migration and impaired resistance to *Leishmania donovani* infection in mice deficient in CCL19 and CCL21. *J. Immunol.* 176: 5486–5493.
40. Ato, M., S. Stäger, C. R. Engwerda, and P. M. Kaye. 2002. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat. Immunol.* 3: 1185–1191.
41. Steigerwald, M., and H. Moll. 2005. *Leishmania major* modulates chemokine and chemokine receptor expression by dendritic cells and affects their migratory capacity. *Infect. Immun.* 73: 2564–2567.
42. Olivier, M., D. J. Gregory, and G. Forget. 2005. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin. Microbiol. Rev.* 18: 293–305.
43. Alexander, J., and P. M. Kaye. 1985. Immunoregulatory pathways in murine leishmaniasis: different regulatory control during *Leishmania mexicana mexicana* and *Leishmania major* infections. *Clin. Exp. Immunol.* 61: 674–682.
44. Buxbaum, L. U., H. Denise, G. H. Coombs, J. Alexander, J. C. Mottram, and P. Scott. 2003. Cysteine protease B of *Leishmania mexicana* inhibits host Th1 responses and protective immunity. *J. Immunol.* 171: 3711–3717.
45. Buxbaum, L. U., J. E. Uzonna, M. H. Goldschmidt, and P. Scott. 2002. Control of New World cutaneous leishmaniasis is IL-12 independent but STAT4 dependent. *Eur. J. Immunol.* 32: 3206–3215.
46. Carvalho, E. M., D. Correia Filho, O. Bacellar, R. P. Almeida, H. Lessa, and H. Rocha. 1995. Characterization of the immune response in subjects with self-healing cutaneous leishmaniasis. *Am. J. Trop. Med. Hyg.* 53: 273–277.